MAMMALIAN DNA BINDING MEMBRANE-ASSOCIATED PROTEIN-ENCODING GENE AND USES

This application claims priority to U.S. Provisional Application Serial No. 60/222,624, filed August 1, 2000, the entirety of which is incorporated by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to cell membrane-associated DNA binding proteins (termed DNA-R herein) from mammalian species and the genes corresponding to such receptors. Specifically, the invention relates to the isolation, cloning and sequencing of complementary DNA (cDNA) copies of messenger RNA (mRNA) encoding a novel mammalian DNA-R gene. The invention also relates to the construction of recombinant expression constructs comprising cDNA of this novel DNA-R gene, said recombinant expression constructs being capable of expressing DNA-R protein in cultures of transformed prokaryotic and eukaryotic cells. Production of the receptor protein in such cultures is also provided, as well as the production of fragments thereof having biological activity. The invention relates to the use of such cultures of such transformed cells to produce homogeneous compositions of the novel DNA-R protein. The invention also provides cultures of such cells producing this DNA-R protein for the characterization of novel and useful drugs. Antibodies against and epitopes of this novel DNA-R protein are also provided by the invention.

2. Background of the Invention

Extracellular DNA is a potent biological signal, being capable of initiating a wide range of immune responses *in vivo* and *in vitro*, including cytokine production, influx of neutrophils, IgM secretion, B-cell proliferation and enhanced natural killer activity. These properties of extracellular DNA enable naked DNA to be used as vaccines, in some instances. In addition, extracellular DNA has been used to introduce new genetic information into cells, both *in vivo* and *in vitro*.

One important aspect of extracellular DNA transfer into mammalian cells is gene therapy. Gene transfer therapy offers the potential for treatment of a variety of diseases. The ability to provide safe, efficient, and selective *in vivo* gene delivery will be a critical component of future protocols. Gene transfer by injection of either plasmid DNA or DNA/liposome

complexes has been demonstrated to be safe and permits expression of gene products. The uptake of DNA/liposome complexes does not depend upon specific cell-surface receptors while the mechanism mediating uptake of plasmid DNA by cells remains unknown.

In order to realize the full potential of this technology, safe delivery and efficient transgene expression of DNA in selected tissues and cells must be achieved. One approach to target DNA to tissue is the use of a receptor-mediated mechanism for the binding and internalization of DNA. Viral (retrovirus, adenovirus, adeno-associated virus) delivery of DNA to cells is via a receptor-mediated mechanism, however this technique has limited *in vivo* clinical application. Viral vectors have been most frequently used for *ex vivo* gene therapy, but the technical problems associated with transplanting transduced cells remain a serious obstacle. In addition, viral vectors have the potential to lead to virus infection or to induce an immune response against antigenic viral coat proteins.

Non-viral methods of gene delivery include liposomes, the so-called "gene gun", and direct injection. Gene transfer with liposomes has been shown to result in uptake and expression of DNA. Although DNA/liposomes are effectively taken up and the cDNA on the plasmid expressed, the process is believed to be nonspecific with limited possibility of targeting selected tissue. An alternative is to administer plasmid DNA directly, without a delivery system. Cells lines in tissue culture have demonstrated *in vitro* uptake of plasmid DNA and the expression of the transgene on the plasmid. It has also been shown that DNA, injected directly *in vivo,* has been taken up and the encoded genes have been expressed . While this approach has been shown to be a safe and free from problems associated with DNA delivery by viruses, the therapeutic potential of this technology is often limited by poor transgene expression from plasmid DNA in many tissues. In addition, the mechanism by which plasmid DNA is bound and internalized into cells is not well established. Knowledge of the mechanism of plasmid DNA binding to the cell surface, and how DNA is internalized and expressed, will be critical to enhancing transgene methods that also have the potential to target selected tissues.

Antisense oligonucleotides (ODN) are another form of extracellular DNA of great importance. ODN are considered potential therapeutic agents against various pathogens and oncogenes due to their ability to specifically inhibit gene expression. When injected into tissues, ODN are internalized by cells and bind to complementary region of mRNA to inhibit translation of proteins in a highly specific manner. Different antisense ODN to HIV RNA have been shown to inhibit the infectivity of the virus in cultured human leukemia cells. Although human clinical trials using ODN to treat AIDS and other diseases are ongoing, the lack of a

precise understanding of where and how gene expression is effected hinders the optimization of this technique.

Extracellular DNA is also associated with human diseases, such as cystic fibrosis. Cystic fibrosis (CF) is the most common lethal genetic disease in North America. It affects one in 2500 live births and affected individuals have a median life expectancy of 28 years (Davis *et al.*, 1996, *Amer. J. Respir. Crit Care Med.* 157: 1234-1239). There is a growing body of evidence showing that inflammation, particularly the injurious products of neutrophils, may be responsible for lung damage (Doring, 1997, *Ped. Pulmonol. Supp.* 16: 271-272); it is now recognized that most of the morbidity and over 90% of the mortality results from chronic progressive inflammation of the lungs. Corticosteroids have a broad anti-inflammatory effect, particularly on neutrophils. A multicenter trial showed beneficial effects of oral corticosteroids on lung function. However, adverse effects such as growth retardation, glucose abnormalities and cataracts prelude this treatment as a long-term option (Eigen *et al.*, 1995, *J. Ped.* 126: 515-523). The nonsteroidal anti-inflammatory drug, ibuprofen, has also been studied (Konstan *et al.*, 1995, *N. Engl. J. Med.* 332: 848-854). The drug is beneficial, but continued monitoring is needed to determine the safety of long-term, high dose therapy. Other therapies that treat the injurious products of neutrophils, for example, antiproteases and antioxidants, are currently under investigation (Konstan, 1998, *Clin. Chest Med.* 19: 505-513).

The vicious airway fluid characteristic of CF can obstruct airflow and provides a viable growth medium for pathogenic bacteria, and cell lysis of these bacteria can produce extracellular DNA that causes inflammation. Recombinant human Dnase (rhDNase) has been clinical use since 1994 (Kontsan, 1998, *ibid.*). The rhDNase, administered by inhalation, has been used to cleave the extracelular airway DNA and reduce the viscosity of the airway fluid. Treatment with rhDNase produces a small improvement in lung function (Cramer & Bosso, 1996, *Ann. Pharmacol.* 30: 656-661). However, when treatment is stopped, patients can deteriorate to a point below their previous baseline (Bush, 1998, *Ped. Pulmonol.* 25: 79-82). In addition, a recent report showed that despite improvements in lung function, there were no changes in airway inflammation (Henry *et al.*, 1998, *Ped. Pulmonol.* 26: 97-100). Although the DNA is broken down by the Dnase, it is not entirely degraded, and hydrolized fragments are still potentially immunostimulatory and can contribute to inflammation. Thus rhDNase may be masking the process of on-going lung destruction.

There are also a variety of conventional treatments for CF including physiotherapy, nutritional support and drugs (Bilton & Mahadeva, 1997, *J. Royal Soc. Med.* 90: Suppl.31, 2-5).

Because the events that trigger and sustain inflammation in patients with CF are not clearly understood, a variety of approaches have been developed to treat different components of the disease. Antibiotics, anti-inflammatories, and therapies to reduce the viscosity of the airway fluid are all approaches that are being used and investigated. Aggressive antibiotic therapy has helped the acute control of infection, but rarely if ever are the bacteria in the airways of patients with CF completely eradicated. These pathogenic bacteria chronically stimulate and exacerbate inflammation. Although some of the currently-available treatments can help to alleviate symptoms and slow the progression of disease, none of the current treatments can prevent ultimate respiratory failure.

One important clinical observation is that greatly increased amounts of extracellular DNA, of host and bacterial origin, are present in the airway of patients with cystic fibrosis. Recent investigation has demonstrated that extracellular DNA, purified from sputum of patients with CF, will directly induce inflammation in the mouse lung (Schwartz *et al.*, 1997, *J. Clin. Invest.* 100: 68-73). The DNA purified from the sputum of patients with cystic fibrosis has been shown to be composed primarily of host-derived DNA and only a small fraction appears to be bacterial DNA (Schwartz *et al.*, 1997, *ibid.*). One possible explanation is that extracellular DNA binds to immune lung cells in the lungs and induces the secretion of pro-inflammatory cytokines and neutrophic migration to the lung, leading to severe airway inflammation. Extracellular DNA binding to immune cells in the lung, such as alveolar macrophages are stimulated to produce pro-inflammatory cytokines that recruit and activate neutrophils leading to inflammation. When these neutrophils undergo apoptosis and release their DNA the cycle is repeated and inflammation is maintained or increased. Thus, methods and reagents that block DNA binding to cytokine producing cells may therefore provide better treatment of CF patients than are currently available.

Although there have been several reports in the art that DNA could bind to cell surfaces (Bennett, 1993, *Antisense Res. Develop.* 3: 235-241; Bennett *et al.*, 1986, *J. Rheumatol.* 13: 679-685; Gabor & Bennett, 1984, *Biochem Biophys. Res. Commun.* 122:1034-1039; Hefeneider *et al.*, 1990, *J. Invest. Dermatol.* 94: 79S-84S; Bennett *et al.*, 1987, *J. Exp. Med.* 166: 850-863; Bennett *et al.*, 1991, *Clin. Exp. Immunol.* 86: 374-379; Bennett *et al.*, 1992, *Clin. Exp. Immunol* 90: 428-433; Bennett *et al.*, 1985, *J. Clin.Invest.* 76: 2182-2190; Hefeneider *et al.*, 1992, *Lupus* 1: 167-173; Hefeneider *et al.*, 1992, *Clin. Immunol. Immunopath.* 63: 245-251; Reid & Chalson, 1979, *Intl. Rev. Cytol.* 60: 27-52; Lerner *et al.*, 1971, *Proc. Natl. Acad. Sci. USA* 68: 1212-1216; Pancer *et al.*, 1981, *J. Immunol.* 127: 98-104; Meinke & Goldstein, 1974, *J. Molec. Biol.* 86:

757-773; Sudar *et al.*, 1986, *Cell. Molec. Biol.* 32: 87-91; Gasparro *et al.*, 1990, *Photochem & Photobiol.* 52: 315-321; Emlen *et al.*, 1988, *Amer. J. Pathol.* 133: 54-60), the art lacks an understanding of how cells mediate extracellular DNA binding. Thus, an understanding of the mechanisms by which eukaryotic cells, particularly mammalian cells, take up extracellular DNA would be important in improving a variety of biological processes.

SUMMARY OF THE INVENTION

The present invention relates to the cloning, expression and functional characterization of a mammalian DNA-R gene. The invention comprises nucleic acids having a nucleotide sequence of a novel mammalian DNA-R gene. The nucleic acids provided by the invention comprise a complementary DNA (cDNA) copy of the corresponding mRNA transcribed *in vivo* from the DNA-R genes of the invention. In a preferred embodiment, the mammalian DNA-R is a human DNA-R.. Also provided are the deduced amino acid sequence of the cognate proteins of the cDNAs provided by the invention, methods of making said cognate proteins by expressing the cDNAs in cells transformed with recombinant expression constructs comprising said cDNAs, and said recombinant expression constructs and cells transformed thereby.

This invention in a first aspect provides nucleic acids, nucleic acid hybridization probes, recombinant eukaryotic expression constructs capable of expressing the DNA-Rs of the invention in cultures of transformed cells, and such cultures of transformed eukaryotic cells that synthesize the DNA-Rs of the invention. In another aspect, the invention provides homogeneous compositions of the DNA-R proteins of the invention, homogeneous compositions of fragments of said DNA-R, most preferably a fragment comprising amino acids 1-575 of the DNA-R, as well as fusion proteins between the DNA-R or fragments thereof and, *inter alia*, epitope markers, and membrane preparations from cells expressing the DNA-R proteins of the invention, and also antibodies against and epitopes of the DNA-R proteins or fragments thereof of the invention. The invention in another aspect provides methods for making said homogenous preparations and membrane preparations using cells transformed with the recombinant expression constructs of the invention and expressing said DNA-R proteins thereby. Methods for characterizing the receptor and biochemical properties of these receptor proteins and methods for using these proteins in the development of agents having pharmacological uses related to the DNA-R of the invention are also provided.

In a first aspect, the invention provides a nucleic acid having a nucleotide sequence encoding a mammalian DNA-R. In a first preferred embodiment, the nucleic acid encodes a

human DNA-R. In this embodiment of the invention, the nucleotide sequence comprises 4351 nucleotides of human DNA-R cDNA comprising 3576 nucleotides of coding sequence, 601 nucleotides of 5′ untranslated sequence and 177 nucleotides of 3′ untranslated sequence. In this embodiment of the invention, the nucleotide sequence of the DNA-R is the nucleotide sequence depicted in Figure 1 (SEQ ID No:1). The sequence shown in Figure 1 will be understood to represent one specific embodiment of a multiplicity of nucleotide sequences that encode the human DNA-R amino acid sequence of 1192 amino acids (SEQ ID No.: 2) of the invention and that these different nucleotide sequences are functionally equivalent and are intended to be encompassed by the claimed invention. In addition, it will be understood that different organisms and cells derived therefrom express preferentially certain transfer RNAs (tRNAs) corresponding to subsets of the degenerate collection of tRNAs capable of encoding certain of the naturally-occurring amino acids, and that embodiments of the multiplicity of nucleotide sequences encoding the amino acid sequence of the human DNA-R protein of the invention that are optimized for expression in specific prokaryotic and eukaryotic cells are also encompassed by the claimed invention. Isolated nucleic acid derived from human genomic DNA and isolated by conventional methods using the human cDNA provided by the invention is also within the scope of the claimed invention. Finally, it will be understood that allelic variations of the human DNA-R, including naturally occurring and *in vitro* modifications thereof are within the scope of this invention. Each such variant will be understood to have essentially the same amino acid sequence as the sequence of the human DNA-R disclosed herein.

Mammalian DNA-R proteins corresponding to the human cDNA of the invention are a second aspect of the claimed invention. In a first embodiment, the mammalian DNA-R protein is a human DNA-R having a deduced amino acid sequence shown in Figure 1 (SEQ ID No.:2). In a second embodiment is provided said human DNA-R protein comprising a membrane preparation from a cell, most preferably a recombinant cell, expressing a nucleic acid encoding a human DNA-R of the invention.

As provided in this aspect of the invention is a homogeneous composition of a mammalian DNA-R having a molecular weight of about 150kD or derivative thereof that is a human DNA-R having an amino acid sequence shown in Figure 1 and identified by SEQ ID No.:2, said size being understood to be the predicted size of the protein before any post-translational modifications thereof. Also provided is a homogeneous composition of An amino-terminal fragment of the human DNA-R comprising amino acid residues 1-575 of the sequence identified as SEQ ID No.: 2. Species of the protein genetically engineered to lack the

transmembrane region of the DNA-R as described herein, and thereby providing soluble forms of the DNA-R of the invention, are also within the scope of this aspect of the invention and are provided herein.

This invention provides both nucleotide and amino acid probes derived from the sequences herein provided. The invention includes probes isolated from either cDNA or genomic DNA, as well as probes made synthetically with the sequence information derived therefrom. The invention specifically includes but is not limited to oligonucleotide, nick-translated, random primed, or *in vitro* amplified probes made using cDNA or genomic clone of the invention encoding a mammalian DNA-R or fragment thereof, and oligonucleotide and other synthetic probes synthesized chemically using the nucleotide sequence information of cDNA or genomic clone embodiments of the invention.

It is a further object of this invention to provide such nucleic acid hybridization probes to determine the pattern, amount and extent of expression of the DNA-R gene in various tissues of mammals, including humans. It is also an object of the present invention to provide nucleic acid hybridization probes derived from the sequences of mammalian DNA-R genes of the invention to be used for the detection and diagnosis of genetic diseases. It is an object of this invention to provide nucleic acid hybridization probes derived from the nucleic acid sequences of the mammalian DNA-R genes herein disclosed to be used for the detection of novel related receptor genes.

The present invention also includes synthetic peptides made using the nucleotide sequence information comprising the cDNA embodiments of the invention. The invention includes either naturally occurring or synthetic peptides which may be used as antigens for the production of DNA-R-specific antibodies, or useful as competitors of DNA-R molecules for nucleic acid binding, or to be used for the production of inhibitors of nucleic acid binding to such DNA-R molecules.

The present invention also provides antibodies against and epitopes of the mammalian DNA-R molecules of the invention. It is an object of the present invention to provide antibodies that are immunologically reactive to the DNA-Rs of the invention. It is a particular object to provide monoclonal antibodies against these DNA-Rs. Hybridoma cell lines producing such antibodies are also objects of the invention. It is envisioned at such hybridoma cell lines may be produced as the result of fusion between a non-immunoglobulin producing mouse myeloma cell line and spleen cells derived from a mouse immunized with a cell line which expresses anti[illegible] or epitopes of a mammalian DNA-R of the invention. The present invention also

provides hybridoma cell lines that produce such antibodies, and can be injected into a living mouse to provide an ascites fluid from the mouse that is comprised of such antibodies. It is a further object of the invention to provide immunologically-active epitopes of the mammalian DNA-R proteins of the invention. Chimeric antibodies immunologically reactive against the DNA-R proteins of the invention are also within the scope of this invention.

The present invention provides recombinant expression constructs comprising a nucleic acid encoding a mammalian DNA-R of the invention wherein the construct is capable of expressing the encoded DNA-R in cultures of cells transformed with the construct. A preferred embodiment of such constructs comprises a human DNA-R cDNA depicted in Figure 1 (SEQ ID No.:1), such constructs being capable of expressing the human DNA-R encoded therein in cells transformed with the construct. Also provided are recombinant expression constructs encoding fragments of said DNA-R, most preferably an amino-terminal fragment comprising amino acid residues 1-575 and fragments genetically engineered to lack the transmembrane domain of said DNA-R, there by providing for production of soluble forms of the DNA-R. In alternative embodiments, the recombinant expression construct encodes a DNA-R fused to epitope sequences recognized by conventional antibodies known in the art. In each instantce, the recombinant expression constructs of the invention are capable of expressing the human DNA-R encoded therein or fragment thereof in cells transformed with the construct.

The invention also provides prokaryotic and more preferably eukaryotic cells transformed with the recombinant expression constructs of the invention, each such cells being capable of and indeed expressing the mammalian DNA-R or fragment or epitope-modified species encoded in the transforming construct, as well as methods for preparing mammalian DNA-R proteins using said transformed cells.

The present invention also includes within its scope protein preparations of prokaryotic and eukaryotic cell membranes containing the DNA-R protein of the invention, or fragment or epitope-modified species thereof, derived from cultures of prokaryotic or eukaryotic cells, respectively, transformed with the recombinant expression constructs of the invention.

The invention also provides methods for screening compounds for their ability to inhibit, facilitate or modulate the biochemical activity of the mammalian DNA-R molecules of the invention, in particular nucleic acid binding thereto. In preferred embodiments, the methods of the invention relate to binding of DNA, particularly double-stranded DNA, and oligonucleotides. The methods of the invention are particularly directed towards identifying compounds that influence DNA or oligonucleotide uptake into cells expressing the DNA-R. In

preferred embodiments, the compounds identified by the methods of the invention influence DNA or oligonucleotide uptake by pinocytosis or endocytosis. In preferred embodiments, the compounds influence DNA or oligonucleotide uptake by increasing the amount of DNA or oligonucleotide that reaches the nucleus of the cell in a form that can be expressed therein. Preferred compounds of the invention are identified by detecting increased uptake or increased expression of a gene, most preferably a reporter gene, encoded by said DNA. In preferred embodiments, cells transformed with a recombinant expression construct of the invention are contacted with such a compound, and the amount of DNA or oligonucleotide taken up by the cell, or the frequency or amount of gene expression, most preferably reporter gene expression, in the cell is assayed.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

DESCRIPTION OF THE DRAWINGS

An understanding of the invention is facilitated by reference to the drawings.

Figure 1 is a schematic diagram illustrating cloning of the cDNA for the DNA-R of the invention. Antisera from a patient with systemic lupus erythroblastosis (SLE) and that inhibits cell surface DNA binding was used to screen λgt11 library from peripheral blood mononuclear cells. A positive clone (clone #88) containing an open reading frame (ORF) was obtained; the open reading frame remained open at the 5' end of the clone. Analysis of the nucleotide sequence of the clone identified a transmembrane region on the 3' end of the clone. A 731bp probe from was used to screen a λgt11 cDNA library made from Raji cell line (human lymphoma cell line). Clone 97D42 which contained 462bp of additional 5' ORF sequence was obtained from this clone. A modification of the polymerase chain reaction (5'random amplification of cDNA ends (RACE- PCR) was used to obtain the remainder of the 5' sequences from HeLa (human cervix carcinoma) and MOLT-4 (human lymphoblastic leukemia) cell lines. This sequence was compiled to produce an ORF of 3543bp that encoded a protein with a calculated molecular weight of 130.5kDa.

Figures 2A and 2B show Northern analysis of human cancer cell lines (Figure 2A) and human tissues (Figure 2B). A 442 bp DNA fragment (probe 11) from the 3' end of the gene coding for DNA-R was used as the radiolabeled probe for each blot.

Figure 3 is a schematic diagram of the human DNA-R of the receptor showing the location of the RING finger, zinc finger, proline rich and hydrophobic regions. An * denotes the N-linked glycosylation sites at amino acid positions 122, 394, 430, 451, 466, 468 and 1150.

Figure 4A is an alignment of conserved cysteines, histidines and aspartic acids of the C3HC3D RING finger in *Homo sapiens* ARD1 GTP-binding protein (Gene Bank Accession 422756). *H. sapiens* CART1 protein (Gene Bank Accession No. _____), *H. sapiens* SBBI03 hypothetical protein (Gene Bank Accession 5032071), *Caenorhabditis elegans* cDNA EST (Gene Bank Accession 3879246), *C. elegans* hypothetical 25.8 KD protein (Gene Bank Accession 2496825) *C. elegans* cDNA EST (Gene Bank Accession 3878739).

Figure 4B is an alignment of conserved cysteines and histidines of the C3H type zinc finger in *C. elegans* PIE-1 (Gene Bank U62896), *Drosophila melanogaster* DTIS 11 (Gene Bank U13397), *H. sapiens* TIS11B Buryrate response factors (EFT-Response factor) (Gene Bank X79066), *Saccharomyces cerevisiae* CTH1 Zinc finger protein (Gene Bank L42133).

Figure 5 is an analysis of DNA-R protein expression in mammalian cells. DNA-R was immunoprecipitated both as the native molecule and in an HA-tagged embodiment from stably-transfected human 293 cells (DNA-R/flu cells). Lane 1, lysate of 293 cells; lane 2, lysate of 293-DNA-R/flu cells; lanes 3-6, immunoprecipitation of 293-DNA-R/flu cell lysates with: rabbit preimmune serum (lane 3) or anti-DNA-R (lane 4), control mouse monoclonal antibody (lane 5) or anti-HA (lane 6). Detected by Western blotting with rabbit anti-DNA-R IgH, immunoprecipitating rabbit IgG heavy chain.

Figure 6 shows the intracellular location of the DNA-R protein, associated with cell membranes in 293-MNAB/flu cells as detected by Western blotting with anti-HA (left half) or anti-DNA-R (right half). T, Triton X-100 whole-cell lysate; M, crude membrane fraction; C, cytosolic fraction.

Figure 7 illustrates immunofluorescence staining using anti-DNA-R and anti-transferrin antibodies on fixed, permeablized A549 cells. A, Double stained with rabbit and sheep nonimmune sera. B, anti-DNA-R. C, anti-transferrin receptor. D, double stained for anti-DNA-R (red) and anti-transferrin receptor (green); colocalized staining appears yellow.

Figure 8A shows the results of antibody staining of 293 cell surface using polyclonal rabbit antisera raised against an amino-terminal fragment (aas 1-575) of the DNA-R of the invention. 293 cells were incubated with preimmune serum (black bars) or immune serum (white bars). Antibody binding was detected with FITC conjugated goat anti-rabbit IgG by flow cytometry. Each bar represents the geometric mean fluorescence intensity $\pm$ sd (n=3, 10,000 viable cells in each analysis). The geometric mean fluorescence of the secondary antibody alone was 7.6 $\pm$ .08 (n=3).

Figure 8B shows antibody binding to the cell surface of Raji and 293 cells using antibodies raised against an amino-terminal fragment (1-575). Cells (Raji, gray bars; 293, white bars) were incubated with serial dilutions of rabbit antisera (#41 bleed 2) produced against the amino terminal portion (amino acids 1-575) of the DNA receptor. The cells were then incubated with FITC-goat anti-rabbit IgG and the fluorescence intensity measure by FACS. Fluorescence due to preimmune sera has been subtracted.

Figure 9A is a schematic diagram of the preparation of the soluble DNA-R protein from the full length DNA-R.

Figure 9B is an SDS-PAGE analysis of expression, affinity purification, and proteolysis of a fusion protein (termed GST/DNA-R) created between glutathione-S-transferase (GST) and an amino-terminal fragment (1-575) of the DNA-R of the invention. Lane 1, whole cell extract of *E. coli* expressing GST/DNA-R; lane 2, GST/DNA-R bound to glutathione (GSH)-sepharose; lane 3, site-specific proteolysis of GST/DNA-R while bound to GSH-sepharose; lane 4, eluate from GSH-sepharose following on-gel proteolysis of GST/DNA-R containing highly purified DNA-R (1-575) peptide.

Figures 10A through 10E show that GST/DNA-R but not control proteins immobilized on glutathione sepharose bind a fluorescently-labeled plasmid DNA (YOYO/pGEM4Z). Shown

in the figure is glutathione sepharose without immobilized protein (Figure 10A), or with immobilized GST (Figure 10B), GST/HST.1 (Figure 10C), GST/CBD (Figure 10D), or GST/DNAR (Figure 10E) incubated with YOYO/pGEM4Z for 30 min at 4° C. After washing, the fluorescence intensity was measured by FACS.

Figures 11A and 11B is the result of a "Southwestern" blot of nucleic acid binding to DNA-R. The experiments were performed by SDS-PAGE analysis, transfer to nitrocellulose and then incubated with (Figure 11A) or without (Figure 11B) biotinylated DNA, followed by binding to streptavidin conjugated with horse radish peroxidase (HRP) and reaction with colorimetric substrate. Absorbance was measured at 450 nm. These results showed that GST/DNA-R but not GST bound biotinylated DNA.

Figure 12 shows the results of an enzyme linked immunosorbent assay (ELISA) in which purified DNA-R fragment (1-575) bound to immobilized DNA. DNA-R fragment (at concentrations of 0, 1, and 10 μ/mL) was incubated with immobilized plasmid DNA in ELISA plates. The plates were then incubated with anti-DNA antibodies at a 1:100 dilution, followed by secondary antibody conjugated to HRP and reaction with colorimetric substrate. Absorbance was measured at 450 nm.

Figure 13 shows that a DNA-R fragment comprising a zinc finger domain (at amino acids 416-435) participates in DNA binding by GST/DNA-R(1-575). Cysteine residues (at positions 416 and 431) were independently altered to serine (and termed C416S and C431S, respectively) or alanine (and termed C416A and C431A, respectively) and ELISA performed to evaluate DNA binding. The zinc finger cysteines at either 416 or 431 were altered to either a serine (C416S, C431S) or an alanine (C416A, C431A). Binding (100ng/well) of wild-type (GST/DNA-R) or mutant GST-fusion proteins or GST alone to immobilized DNA was detected using anti-GST by ELISA. Data are the mean ± s.d. of triplicate determinations.

Figure 14 illustrates results of experiments demonstrating that a soluble form of the DNA-R of the invention binds DNA with high affinity using a nitrocellulose filter binding assay. Soluble DNA-R (sDNA-R) at a concentration of 2 nM and labeled DNA (200 pM, 1 x 10^6 cpm/pmol) held constant and increasing concentrations of unlabeled DNA. Data are the mean ± s.d. of triplicate determinations. Inset: Scatchard transformation of the binding data.

Figure 15 illustrates competitive binding of plasmid DNA with soluble DNA-R. All samples have 0.25nM (YOYO labeled) pGEM-DNA. The diagonal bars have varying amounts of soluble DNA-R added to block DNA binding. The horizontal line bar is control GST protein.

Figure 16 are the results of fluorescence activated cell sorting (FACS) experiments illustrating cell-surface binding of YOYO labeled plasmid DNA in A549 cells. Cells were incubated in the presence (dashed line) and absence (solid line) of 5 μg/mL YOYO-labeled pGEM4Z DNA. The geometric mean fluorescence of untreated and treated cells is 13 and 34 respectively. The difference between the two values (21) is the increase in fluorescence intensity due to YOYO/pGEM4Z. This method of analysis is used for all YOYO/DNA binding analyses by FACS in subsequent figures.

Figures 17A and 17B are the results of FACS analysis of YOYO labeled plasmid DNA binding to A549 cells in the presence of excess unlabeled DNA. Cell surface binding of YOYO/DNA to A549 cells. In Figure 17A (left panel), A549 cells were incubated with YOYO/pGEM4Z in the presence (solid line) and absence (dashed line) of a 25-100 fold excess calf thymus DNA for 2 hr at 4°C. In Figure 17B (right panel), specific DNA binding to A549 cells is shown as the difference in fluorescence intensity of YOYO/pGEM4Z bound to the cells using the data from the left panel,. Data are the mean ± SEM of 4-9 determinations.

Figure 18 shows the effects of trypsin treatment on DNA binding to A549 cells. A549 cells were incubated at 4° C for 30 min with YOYO/pGEM4Z (0.5 to 4 μg/ml). After incubation the cells were washed (white bars) or trypsinized and washed (gray bars), then the fluorescence intensity was measured by FACS. The data are the mean ± SD of triplicate determinations. Trypsin treatment was found to remove most cell-surface DNA binding.

Figure 19 shows the results of experiments indicating that DNA binding to the DNA-R of the receptor is calcium dependent. B16 cells were incubated with 1 μg/mL YOYO/pGEM4Z in PBS with (white bar) or without (gray bar) 1 mM$CaCl_2$, then the fluorescence intensity was measured by FACS. The data are mean ± SD of triplicate measurements.

Figure 20 shows a time course of plasmid DNA uptake by cells expressing the DNA-R of the invention. B16 cells were incubated at 37° C with YOYO/pEGFP-N1 (0.6 to 12 μg/mL) for 1 hr (solid circles), 3 hr (open circles), or 5 hr (solid squares). The cells were then trypsinized to remove DNA bound to the cell-surface, and fluorescence intensity was measured by FACS.

Figure 21 shows that excess unlabeled DNA blocks internalization of YOYO/DNA by A549 cells. Cells were incubated for 2 hr at 37° C with YOYO/pGEM4Z (1 to 25 μg/mL) in the presence (solid line) and absence (dashed line) of a 25- to 100- fold excess of calf thymus DNA. The cells were then washed and the fluorescence measured by FACS. Data are the mean ± SEM of 5 experiments.

Figure 22 illustrates that internalization of plasmid DNA in cells expressing the DNA-R of the invention is temperature dependent. B16 cells were incubated with YOYO/pGEM4Z (12 μg/mL) at 4° C (white bars) or 37° C (gray bars) for the indicated times. The cells were then trypsinized to remove cell-surface bound plasmid, washed, and the fluorescence measured by FACS. The data are the mean ± SD of triplicate determinations.

Figures 23A and 23B show that cell-surface DNA binding to the DNA-R of the invention is related to DNA uptake. 293 cells (solid circles) and G361 cells (open circles) were incubated for 3.5 hr with YOYO/pGEM4Z (at concentrations of from 0.3 to 2.5 μg/mL) at 4° C for binding (Figure 23A, left panel) or 37°C for uptake (Figure 23B, right panel). The cells were then washed (binding) or trypsinized and washed (uptake), and the fluorescence measured by FACS.

Figure 24 shows expression in B16 cells of green fluorescence protein (GFP) transgene from plasmid DNA. Cells were incubated with 12 μg/mL pEGFP-N1 for 6 hr. After 48 hr the cells were trypsinized and fluorescence measured by FACS. Control cells incubated without DNA and cells treated with pEGFP-N1 without carrier showed similar fluorescence, whereas cells incubated with pEGFP-N1 complexed with lipofectamine showed increased fluorescence.

Figure 25 show that nocodazole increases GFP transgene expression from plasmid DNA in A549 cells. A549 cells were incubated for 5 hr at 37° C with 25 μg/mL of pEGFP-N1 in the presence (dashed line) and absence (solid line) of 33 μM nocodazole. After 24 hr the cells were trypsinized and fluorescence measured by FACS.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "mammalian DNA-R" as used herein refers to proteins consisting essentially of, and having substantially the same biological activity as, the protein encoded by the amino acid depicted in Figure 1 (SEQ ID No.:2). This definition is intended to encompass natural allelic variations in the disclosed DNA-R. Cloned nucleic acid provided by the present invention may encode DNA-R protein of any species of origin, including, for example, mouse, rat, rabbit, cat, and human, but preferably the nucleic acid provided by the invention encodes DNA-Rs of mammalian, most preferably human, origin.

The nucleic acids provided by the invention comprise DNA or RNA having a nucleotide sequence encoding a mammalian DNA-R. Specific embodiments of said nucleic acids are depicted in Figure 1 (SEQ ID No.:1), and include any nucleotide sequence encoding a mammalian DNA-R having an amino acid sequence as depicted in Figure 1 (SEQ ID No.: 2). Nucleic hybridization probes as provided by the invention comprise any portion of a nucleic acid of the invention effective in nucleic acid hybridization under stringency conditions sufficient for specific hybridization. Mixtures of such nucleic acid hybridization probes are also within the scope of this embodiment of the invention. Nucleic acid probes as provided herein are useful for isolating mammalian species analogues of the specific embodiments of the nucleic acids provided by the invention. Nucleic acid probes as provided herein are also useful for detecting mammalian DNA-R gene expression in cells and tissues using techniques well-known in the art, including but not limited to Northern blot hybridization, *in situ* hybridization and Southern hybridization to reverse transcriptase - polymerase chain reaction product DNAs. The probes provided by the present invention, including oligonucleotides probes derived therefrom, are also useful for Southern hybridization of mammalian, preferably human, genomic DNA for screening for restriction fragment length polymorphism (RFLP) associated with certain genetic disorders.

The production of proteins such as mammalian DNA-R from cloned genes by genetic

engineering means is well known in this art. The discussion which follows is accordingly intended as an overview of this field, and is not intended to reflect the full state of the art.

Nucleic acid encoding a DNA-R may be obtained, in view of the instant disclosure, by chemical synthesis, by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, or by combinations of these procedures, in accordance with known procedures as illustrated below. Screening of mRNA or genomic DNA may be carried out with oligonucleotide probes generated from the nucleic acid sequence information from mammalian DNA-R nucleic acid as disclosed herein. Probes may be labeled with a detectable group such as a fluorescent group, a radioactive atom or a chemiluminescent group in accordance with know procedures and used in conventional hybridization assays, as described in greater detail in the Examples below. In the alternative, mammalian DNA-R nucleic acid sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, using PCR oligonucleotide primers corresponding to nucleic acid sequence information derived from a DNA-R as provided herein. *See* U.S. Patent Nos. 4,683,195 to Mullis *et al.* and 4,683,202 to Mullis.

Mammalian DNA-R protein may be synthesized in host cells transformed with a recombinant expression construct comprising a nucleic acid encoding the DNA-R nucleic acid, comprising genomic DNA or cDNA. Such recombinant expression constructs can also be comprised of a vector that is a replicable DNA construct. Vectors are used herein either to amplify DNA encoding a DNA-R and/or to express DNA encoding a DNA-R gene. For the purposes of this invention, a recombinant expression construct is a replicable DNA construct in which a nucleic acid encoding a DNA-R is operably linked to suitable control sequences capable of effecting the expression of the DNA-R in a suitable host.

The need for such control sequences will vary depending upon the host selected and the transformation method chosen. Generally, control sequences include a transcriptional promoter, an optional operator or enhancer sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control the termination of transcription and translation. Amplification vectors do not require expression control domains. All that is needed is the ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants. *See*, Sambrook *et al.*, 2001, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Press: New York).

Vectors useful for practicing the present invention include plasmids, viruses (including phage and mammalian DNA and RNA viruses), retroviruses, and integratable DNA fragments

(*i.e.*, fragments integratable into the host genome by homologous recombination). The vector can replicate the gene of interest and function independently of the host genome, or can, in some instances, integrate into the genome itself. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. Transformed host cells are cells which have been transformed or transfected with recombinant expression constructs made using recombinant DNA techniques and comprising nucleic acid encoding a DNA-R protein. Preferred host cells are HEK293 cells, COS-7 cells (Gluzman, 1981, Cell 23: 175-182) and Ltk$^-$ cells. Transformed host cells may express the DNA-R protein, but host cells transformed for purposes of cloning or amplifying nucleic acid hybridization probe DNA need not express the receptor. When expressed, the DNA-R of the invention will typically be located in the host cell membrane. Accordingly, the invention provides preparations of said cell membranes comprising the DNA-R protein of the invention, as well as purified, homogeneous preparations of the receptor protein itself. *See*, Sambrook *et al.*, *ibid.*

Cultures of cells derived from multicellular organisms are a desirable host for recombinant DNA-R protein synthesis. In principal, any higher eukaryotic cell culture is useful, whether from vertebrate or invertebrate culture. However, mammalian cells are preferred, as illustrated in the Examples. Propagation of such cells in cell culture has become a routine procedure. *See* Tissue Culture, Academic Press, Kruse & Patterson, editors (1973). Examples of useful host cell lines are human embryonic kidney (HEK) 293 cells, VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, mouse Ltk$^-$ cell lines and WI138, BHK, COS-7, CV, and MDCK cell lines. HEK293 cell, COS-7 cells and Ltk$^-$ cells are preferred.

The invention provides homogeneous compositions of mammalian DNA-R protein produced by transformed eukaryotic cells as provided herein. Each such homogeneous composition is intended to be comprised of a DNA-R protein that comprises at least 75%, more preferably at least 80%, and most preferably at least 90% of the protein in such a homogenous composition; in said homogeneous preparations, individual contaminating protein species are expected to comprise less than 5%, more preferably less than 2% and most preferably less than 1% of the preparation. The invention also provides membrane preparations from cells expressing mammalian DNA-R protein as the result of transformation with a recombinant expression construct, as described herein. Also specifically provided by the invention are fragments of the DNA-R of the invention, most preferably DNA binding fragments thereof. In preferred embodiments, said fragments include soluble forms of the receptor lacking the transmembrane domain and an amino-terminal fragment (most preferably amino acids 1-575)

comprising zinc finger and RING sequence motifs known in the art to be related to DNA-protein binding.

Mammalian DNA-R proteins made from cloned genes in accordance with the present invention may be used for screening compounds that effect DNA binding to cells *in vivo* and *in vitro*, as more fully described herein, and that affect DNA uptake and expression of genes encoded thereby. For example, host cells may be transformed with a recombinant expression construct of the present invention, a mammalian DNA-R expressed in those host cells, and the cells or membranes thereof used to screen compounds for their effect on DNA binding. By selection of host cells that do not ordinarily express a DNA-R, pure preparations of membranes containing the receptor can be obtained.

The recombinant expression constructs of the present invention are useful in molecular biology to transform cells which do not ordinarily express a DNA-R to thereafter express this receptor. Such cells are useful as intermediates for making cell membrane preparations useful for receptor binding activity assays, which are in turn useful for drug screening. The recombinant expression constructs of the present invention thus provide a method for screening potentially useful drugs at advantageously lower cost than conventional animal screening protocols. While not completely eliminating the need for ultimate *in vivo* activity and toxicology assays, the constructs and cultures of the invention provide an important first screening step for the vast number of potentially useful drugs synthesized, discovered or extracted from natural sources each year. This utility thereby enables rational drug design of novel therapeutically-active drugs using currently-available techniques (*see* Walters, "Computer-Assisted Modeling of Drugs", *in* Klegerman & Groves, eds., 1993, Pharmaceutical Biotechnology, Interpharm Press:Buffalo Grove, IL, pp. 165-174).

The recombinant expression constructs of the present invention may also be useful in gene therapy. Cloned genes of the present invention, or fragments thereof, may also be used in gene therapy carried out homologous recombination or site-directed mutagenesis. *See generally* Thomas & Capecchi, 1987, Cell 51: 503-512; Bertling, 1987, Bioscience Reports 7: 107-112; Smithies *et al.*, 1985, Nature 317: 230-234.

Nucleic acid and oligonucleotide probes as provided by the present invention are useful as diagnostic tools for probing DNA-R gene expression in tissues of humans and other animals. For example, tissues are probed *in situ* with oligonucleotide probes carrying detectable groups by conventional autoradiographic or other detection techniques, to investigate native expression of this receptor or pathological conditions relating thereto. Further, chromosomes can be probed

to investigate the presence or absence of the corresponding DNA-R gene, and potential pathological conditions related thereto. Oligonucleotides, particularly antisense oligonucleotides, are also useful for decreasing expression of the DNA-R in cells that overexpress the receptor or whose expression is disadvantageous in a host organism, either generally or in specific tissues. An example of the latter instance is in airway epithelial cells and macrophages in lung tissues in cystic fibrosis patients, as set forth more fully herein.

The invention also provides antibodies that are immunologically reactive to the DNA-R protein or epitopes thereof provided by the invention. The antibodies provided by the invention may be raised, using methods well known in the art, in animals by inoculation with cells that express a DNA-R or epitopes thereof, cell membranes from such cells, whether crude membrane preparations or membranes purified using methods well known in the art, or purified preparations of proteins, including protein fragments and fusion proteins, particularly fusion proteins comprising epitopes of the DNA-R protein of the invention fused to heterologous proteins and expressed using genetic engineering means in bacterial, yeast or eukaryotic cells, said proteins being isolated from such cells to varying degrees of homogeneity using conventional biochemical methods. Synthetic peptides made using established synthetic methods *in vitro* and optionally conjugated with heterologous sequences of amino acids, are also encompassed in these methods to produce the antibodies of the invention. Animals that are useful for such inoculations include individuals from species comprising cows, sheep, pigs, mice, rats, rabbits, hamsters, goats and primates. Preferred animals for inoculation are rodents (including mice, rats, hamsters) and rabbits. The most preferred animal is the mouse.

Cells that can be used for such inoculations, or for any of the other means used in the invention, include any cell line which naturally expresses the DNA-R provided by the invention, or more preferably any cell or cell line that expresses the DNA-R of the invention, or any epitope thereof, as a result of molecular or genetic engineering, or that has been treated to increase the expression of an endogenous or heterologous DNA-R protein by physical, biochemical or genetic means. Preferred cells are mammalian cells, most preferably cells syngeneic with a rodent, most preferably a mouse host, that have been transformed with a recombinant expression construct of the invention encoding a DNA-R protein, and that express the receptor therefrom.

The present invention also provides monoclonal antibodies that are immunologically reactive with an epitope derived from a DNA-R of the invention, or fragment thereof, present on the surface of such cells or in membrane preparations thereof or used after varying degrees

of biochemical purification. Particularly useful are soluble fragments of the DNA-R of the invention, including for example species of the receptor genetically engineered to remove the transmembrane domain, and amino-terminal fragments, most preferably DNA binding fragments of the receptor. Such antibodies are made using methods and techniques well known to those of skill in the art. Monoclonal antibodies provided by the present invention are produced by hybridoma cell lines, which are also provided by the invention and are made by methods well known in the art.

Hybridoma cell lines are made by fusing individual cells of a myeloma cell line with spleen cells derived from animals immunized with cells expressing a DNA-R of the invention, as described above. The myeloma cell lines used in the invention include lines derived from myelomas of mice, rats, hamsters, primates and humans. Preferred myeloma cell lines are from mouse, and the most preferred mouse myeloma cell line is P3X63-Ag8.653. The animals from whom spleens are obtained after immunization are rats, mice and hamsters, preferably mice, most preferably Balb/c mice. Spleen cells and myeloma cells are fused using a number of methods well known in the art, including but not limited to incubation with inactivated Sendai virus and incubation in the presence of polyethylene glycol (PEG). The most preferred method for cell fusion is incubation in the presence of a solution of 45% (w/v) PEG-1450. Monoclonal antibodies produced by hybridoma cell lines can be harvested from cell culture supernatant fluids from *in vitro* cell growth; alternatively, hybridoma cells can be injected subcutaneously and/or into the peritoneal cavity of an animal, most preferably a mouse, and the monoclonal antibodies obtained from blood and/or ascites fluid.

Monoclonal antibodies provided by the present invention are also produced by recombinant genetic methods well known to those of skill in the art, and the present invention encompasses antibodies made by such methods that are immunologically reactive with an epitope of an amino acid receptor of the invention. The present invention also encompasses antigen-binding fragments, including but not limited to F_v, F(ab) and $F(ab)'_2$ fragments, of such antibodies. Fragments are produced by any number of methods, including but not limited to proteolytic or chemical cleavage, chemical synthesis or preparation of such fragments by means of genetic engineering technology. The present invention also encompasses single-chain antibodies that are immunologically reactive with an epitope of a DNA-R, made by methods known to those of skill in the art.

The present invention also encompasses an epitope of a DNA-R of the invention, comprised of sequences and/or a conformation of sequences present in the receptor molecule.

This epitope may be naturally occurring, or may be the result of chemical or proteolytic cleavage of a receptor molecule and isolation of an epitope-containing peptide or may be obtained by chemical or *in vitro* synthesis of an epitope-containing peptide using methods well known to those skilled in the art. The present invention also encompasses epitope peptides produced as a result of genetic engineering technology and synthesized by genetically engineered prokaryotic or eukaryotic cells.

The invention also includes chimeric antibodies, comprised of light chain and heavy chain peptides immunologically reactive to a DNA-R-derived epitope. The chimeric antibodies embodied in the present invention include those that are derived from naturally occurring antibodies as well as chimeric antibodies made by means of genetic engineering technology well known to those of skill in the art.

Nucleic acids encoding the receptor, the DNA-R and DNA-binding fragments thereof, are advantageously used to modulate expression or activity of the receptor in cells *in vivo* and *in vitro*. As provided herein, the DNA-R of the invention, particularly soluble embodiments thereof, can competitively bind DNA to reduce said binding to cells expressing the DNA-R. DNA binding to the DNA-R in certain cells, such as airway epithelial cells and macrophages in lung, is associated with the activation of inflammatory processes that cause a significant proportion of the morbidity and mortality associated with cystic fibrosis, chronic bronchitis and other chronic lung diseases. Thus, the invention provides a variety of methods for reducing said morbidity and mortality by interfering with DNA binding to cells in the lung. In one embodiment, soluble DNA-R species can be administered, most preferably by aerosol administration using formulations, excipients and vehicles well known in the art, directly to lung tissue, and competitive DNA binding achieved thereby. In alternative embodiments, antisense oligonucleotides can be delivered to lung tissue, most preferably by aerosol administration, and expression of the DNA-R in target cells of the lung repressed thereby. In further alternatives, antibodies, most preferably monoclonal antibodies, that specifically inhibit DNA binding to the DNA-R of the invention can be used to inhibit DNA binding to said lung cells.

The DNA-R of the invention, particularly soluble embodiments and DNA-binding fragments thereof, are also useful in treating other inflammation-associated diseases and conditions, including otitis media, septic arthritis and any bacterial or viral infection that causes inflammation by interaction with the DNA-R

Additionally, the DNA-R of the invention can be used to screen compounds that modulate DNA binding, uptake and expression. Introduction of DNA, particularly DNA

encoding a desired gene, is a methodology well known in the art. However, DNA introduction methods have been developed empirically and without any understanding of the molecular bases of DNA uptake. Specifically, heretofore specific DNA binding to a DNA-R as disclosed herein and uptake thereby by endocytosis was unappreciated in the art. Identification of the DNA-R of the invention thus provides a novel target for developing compounds and methods for increasing efficiency of DNA uptake and expression of genes encoded thereby.

Another advantageous method provided by this invention is the use of DNA-R expressed in tumor cells to facilitate delivery of DNA-binding anticancer drugs to tumor cells. Drugs such as Adriamycin (Doxorubicin) are in clinical use for the treatment of cancer patients. Enhanced extracellular DNA uptake in tumor cells expression the DNA-R of the invention would facilitate uptake of such DNA-binding anticancer drugs by using extracellular DNA as a carrier of the drug into the cell. The association of the drug with the extracellular DNA might enable the drug to avoid active efflux produced in tumor cells, *inter alia*, by drug resistance mediators such as P-glycoprotein. Employing the same rationale as with gene transfer, the selective augmentation of DNA binding receptors on tumor cells would enhance uptake of DNA-binding drugs and result in an increased therapeutic effect. In alternative embodiments, other diseases, such as malaria, can be treated in a similar fashion, based on the development of cell-surface DNA binding in red blood cells parasitized with the malarial parasite *Plasmodium falciparum*.

The Examples which follow are illustrative of specific embodiments of the invention, and various uses thereof. They set forth for explanatory purposes only, and are not to be taken as limiting the invention.

EXAMPLE 1

Isolation of a Human Membrane-Associated DNA Receptor (DNA-R)

As described in the specification above, DNA binding to cells had been observed in the art, and the behavior of said binding suggested the existence of a DNA binding protein expressed at the cell surface. In order to isolate a novel DNA-binding protein from human cells, serum from a patient with systemic lupus erythematosus (SLE), treated to deplete the sera of anti-DNA antibodies by multiple (6X) passages over a DNA sepharose column, was used to screen a λgt11 cDNA expression library made from liposaccharide stimulated human monocytes. This serum has been shown to have anti-DNA receptor activities (defined by the

blocking of DNA binding to cells; Bennet *et al.*, 1992. *J. Clin. Invest.* 76: 2182-2190).

From approximately one million plaques screened with this sera, ten positive phage clones were identified and isolated according to the technique of Young and Davis (1983, *Proc. Natl. Acad. Sci. USA* 80: 1194-1198). The clones were grouped into two classes, based on Southern blot and Western blot analyses using eluted antibodies. Sequence analysis of the 1.4 kilobase (kb) insert of one clone (clone 88), which was highly reactive on Western blots with SLE serum, revealed an open reading frame that was open at the 5' end of the clone and contained a translation stop codon at the 3' end. This open reading frame coded for a 46.7 kiloDalton (kDa) protein fragment.

The full length cDNA for the putative DNA-R was obtained in segments from peripheral blood mononuclear cells, human Burkitt lymphoma cells (Raji; Accession No. CCL 86, American Type Culture Collection, Manassas, VA), human cervical carcinoma cells (HeLa; ATCC Accession No. CCL 2), and human lymphoblastic leukemia cells (MOLT-4; ATCC Accession No. CRL 1582). A 731 base pair (bp) DNA probe from clone 88 was used to screen a λgt11 phage library from a Raji cell line (the library was obtained from Clonetech Labs, Palo Alto, CA). A 2409 bp clone which contained an additional 462 bp of 5' open reading frame (ORF) sequence was obtained from this screening. Additional sequence from the 5' extent of the cDNA was isolated using two variations of the 5' RACE (rapid amplification of cDNA ends) method. In the first 5' RACE method, single stranded DNA (ssDNA) was synthesized from HeLa cell mRNA using polyT primers and reverse transcriptase. A polyA tail was added to the 5' end of the ssDNA by terminal transferase. The single stranded cDNA was amplified using a gene specific primer and a polyT primer. This clone contained 753 bp of additional sequence 5' of the previously obtained sequence. The remainder of the 5' sequence of the cDNA was obtained from MOLT-4 cDNA by Marathon Race cDNA amplification (Clonetech Labs, Palo Alto, CA) according to the manufacturer's instructions. This procedure produced an additional 1290 bp clone consisting of 340 bp of ORF and a 950 bp 5' untranslated region. Combining the results from these screening and amplification experiments produced the predicted full length cDNA encoding the DNA-R of the invention.

A complete, full-length cDNA for the putative DNA receptor was cloned as a single RT-PCR product from MOLT-4 mRNA using oligonucleotide primers having the following sequence:

Primer 5': ACCCGAGCATGGATCCGCCACCATGGCTGTGCAGGCAGC (SEQ ID NO: 5)

and

Primer 3': GGTATCTAGATCCATGGTGTGGTCAC (SEQ ID NO: 6)

The complete sequence was 4351 nucleotides (SEQ ID NO: 1) in length with a defined open reading frame of 3576 nucleotides encoding a protein of 1192 amino acids (SEQ ID NO: 2). The isolation protocol is schematically illustrated in Figure 1.

EXAMPLE 2

DNA Receptor Gene Expression and Protein Sequence Analysis

Tissue-specific and cell line-specific expression patterns of its corresponding mRNA in various human tissues was analyzed by Northern blot analysis on RNA isolated from various tissues and cancer cell lines. The results of these experiments are shown in FIG. 2.

A panel of tissue samples was examined by Northern hybridization analysis performed under low stringency conditions, defined as hybridization at 42°C in 5X SSPE (0.75M NaCl, 0.05M NaH_2PO_4, 5mM EDTA), 10X Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumen), 100 µg/mL salmon sperm DNA, 2% SDS and 50% deionized formamide and 1-2 x 10^6 cpm random-primed, ^{32}P labeled probe, followed by washing in 0.1X SSC (15mM NaCl, 1.5mM trisodium citrate, 0.1% SDS). The blots were hybridized with a probe consisting of 442 bp of sequence from the 3' end of the coding sequence from the DNA-R gene to determine the distribution of receptor mRNA. This analysis revealed two major transcripts of 9.5 and 6.8 kb in all human tissues and cancer cell lines examined. Transcript expression was relatively abundant in spleen, testis, ovary, and small intestine. Several smaller transcript sizes were also observed in some of the tissues and cell lines examined (FIG. 2).

A homology search against human genomic sequence placed the DNA receptor on chromosome 9q34 (GenBank Accession number AC007066, marker HIM9.89 on Contig CHR9.SL27). The genomic sequence, which covered 85% of the cDNA starting from the 5' end, revealed the location of 16 complete exons and the beginning of a 17th exon. A BLAST search of the expressed tag sequence (EST) database indicated wide expression of this gene in normal human tissue (liver/spleen, prostate epithelial, germinal B cell, white adipose, pregnant uterus, fetal heart/liver and spleen) and in tumor and transformed human cells (Jurkat, HL60,

293, G361, B-cell lymphocytic leukemia, colon tumor, melanoma, and parathyroid tumor).

Figure 3 provides a schematic diagram of the structure of the DNA-R protein encoded by SEQ ID No. 1. Hydropathy analysis identified a 38 amino acid hydrophobic region near the carboxy terminus of the protein (amino acids 1133-1171) which is a potential transmembrane domain. Expression of a soluble species of this receptor by deleting these amino acids supported identification of this region as a transmembrane domain. In addition, seven consensus sites for –linked glycolsylation have been identified (amino acid positions 122, 394, 430, 451, 466, 468, and 1150) and there is a proline rich (20% of the residues are proline) region spanning amino acids 549-809 (FIG. **3**). The calculated isoelectric point of the DNA receptor protein is 6.4. The BLAST search also identified two art-recognized amino acid sequence motifs in the DNA-R sequence: a C3HC3D Ring finger subtype located near the amino terminus (amino acids 14-50) and a C3H zinc finger located near the center of the protein sequence (amino acids 416-435). An alignment of several ring finger motifs is shown in FIG. **4A**; DNA-R differs from the originally identified C3HC4 Ring finger motif by the replacement of the last cysteine with an aspartic acid. The alignment of the conserved cysteines and histidines of the C3H zinc finger motif is shown in FIG. **4B**.

EXAMPLE 3
DNA Receptor Expression and Protein Expression Analyses

The DNA-R of the invention was produced recombinantly as follows. A *Bam*HI-*Hpa*I cDNA fragment containing the coding sequence for amino acids 1-1190 (*i.e.*, missing the two most carboxylterminal amino acids) of the DNA-R of the invention was cloned into pTriplFlu (obtained from J. Epstein, University of Pennsylvania, Philadelphia, PA). This vector contains a sequence encoding an epitope tag from the influenza hemagglutinin gene in triplicate inserted immediately 3' of the multiple cloning site of the parent vector, pcDNA3, and which are in-frame with the inserted DNA-R cDNA sequence. This vector was introduced into human 293 cells by transfection using Lipofectamine (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Transfected cells (293-DNA-R/flu) were selected by culturing in growth media (DMEM supplemented with 10% fetal calf serum, 2mM L-glutamine, 100U/mL penicillin and 100µg/mL streptomycin) supplemented with 500 µg/mL G418.

In order to characterize DNA-R protein expression in mammalian cells, immunoprecipitation and Western blotting experiments were performed with protein extracts isolated from several cell lines using polyclonal antisera raised against an amino-terminal

fragment of the DNA-R of the invention, comprising amino acid residues 1-575.

Polyclonal antibodies were produced to a purified fragment of the DNA-R (comprising amino acids 1-575) using conventional techniques. Three female New Zealand White rabbits (Western Oregon Rabbit Company), weighing 2.3-3.0 kg, were injected subcutaneously with 50μg of the DNA-R peptide that was produced in bacteria as a GST fusion protein (described in Example 4) and purified from its fusion partner. The antigen was emulsified with Titre-Max (CytRx Corp., Norcross, GA) in a final volume of 0.5 mL. The rabbits were boosted 4 weeks later with 15 μg of antigen/Titre-Max mixture, again 2 weeks later, and were maintained on a once-a-month booster schedule thereafter. The rabbits were bled 7-10 days after each boost with antigen and the sera analyzed for reactivity to the immunizing antigen.

The polyclonal antisera obtained from the inoculated rabbits was used in Western blot analyses. A protein of Mr ~ 1.5×10^5 was identified by the anti-DNA-R antibody in most cells tested (including 293, COS7, G361, HeLa, HRE605, MOLT-4, Raji, A549, B16). A protein with a similar mobility was detected in lysates of genetically-engineered human 293 cells (293-DNA-R/flu) that were stably transfected with an expression vector for a carboxy-terminal HA-tagged DNA-R (pDNA-R/flu). As shown in FIG. **5**, this protein was detected by immunoprecipitation and/or Western blot analysis with either the rabbit polyclonal anti-DNA-R (1-575) antisera described above or with a mouse monoclonal antibody (anti-HA) specific for the carboxyl-terminal HA tag in the recombinantly-produced protein.

In order to determine cellular localization of the DNA-R protein, crude membrane fractions from recombinant 293-DNA-R/flu cells were examined by Western blot analysis with either anti-DNA-R or anti-HA antibodies. The results shown in FIG. **6** indicated that essentially all the DNA-R protein in those cells was associated with the membrane fraction. Indirect immunofluorescence on fixed, permeabilized cells showed anti-DNA-R staining was predominantly localized to the perinuclear region of the cell, although no nuclear staining was observed (FIG. **7**). Double staining with anti-DNA-R and anti-transferrin receptor antibodies showed partial colocalization of the DNA-R and transferrin receptor, however the DNA-R did not colocalize with the transferrin receptor in peripheral endosomes (FIG. **7**). These results indicate that extracellular DNA is taken up by cells expressing the DNA-R of the invention by endocytosis, and suggest that compounds that influence intracellular trafficking of molecules taken by endocytosis are useful for modulating the intracellular fate (such as degradation in lysosomes or transport to the cell nucleus) of extracellular DNA.

To determine if DNA-R is located on the cell surface, cells were incubated with anti-DNA-R (1-575) immune rabbit serum. Antibody binding was detected by flow cytometry with FITC labeled secondary antibodies to rabbit IgG. At all serum dilutions the fluorescence intensity of the cells incubated with immune serum was significantly higher than that of cells incubated with preimmune serum ($p<0.003$) suggesting that DNA-R is expressed on the cell surface (FIG. **8**).

These results demonstrated that the DNA-R protein, either natively expressed or expressed from the cloned cDNA of the invention, or genetically-engineered embodiments thereof, localized to cell membranes as predicted by the hydropathy plot of the carboxyl terminus.

EXAMPLE 4

Soluble DNA-R Fusion Protein Binds DNA with High Affinity

The capacity of the DNA-R of the invention to bind DNA, and particularly the capacity of a soluble form of the DNA-R protein to bind DNA (which would be useful for the development of a therapeutic agent as described more particularly below) was determined. For these experiments, a fusion protein between the amino terminal portion of the DNA receptor (amino acids 1-575), lacking the transmembrane region but containing both the RING and zinc finger domains, was produced using the pGEX vector system (Pharmacia, Kalamazoo, MI) for expression of glutathione-S-transferase (GST)-fusion proteins in *E. coli* and named GST/DNA-R (1-575). A schematic diagram of the production of this protein fragment and its structure relative to the full-length DNA-R of the invention is shown in Figure 9A. Polyacrylamide gel analysis of the production, proteolysis, and purification of the recombinant DNA-R peptide is shown in FIG. **9B**. The calculated molecular weights of the GST/DNA-R fusion protein and the DNA-R peptide are 90 kDa and 63 kDa respectively.

The purified GST/DNA-R fusion protein was then examined for its ability to bind plasmid DNA. Three independent *in vitro* assays were used to assess DNA binding by the fusion protein. First, the ability of GST/DNA-R, bound to glutathione sepharose beads, to bind YOYO-labeled plasmid DNA was determined by incubation with 0.9 μg YOYO/DNA in 0.5 mL of medium. (YOYO-1 is an intercalating fluorochrome that is flourescent only when bound to DNA, obtained from Molecular Probes, Eugene, OR .) Beads (3.5×10^5) and YOYO/DNA

were incubated for 30 minutes at 4°C, washed once and then fluorescence intensity analyzed by FACS. As seen in FIG. **10**, the GST/DNA-R fusion protein was extremely efficient in binding DNA whereas purified GST protein alone and two additional, unrelated GST-fusion proteins (GST-CBD and GST-HST.1, gifts from Dr. Roland Kwok, Vollum Institute, Portland OR) failed to show any DNA binding capability. Following FACS analysis an aliquot of glutathione sepharose-bound protein from each sample used in the DNA binding assay was analyzed by SDS-PAGE followed by Coomassie blue staining. An approximately equivalent amount of each GST-fusion protein was shown to be present in each sample.

To further assess whether the GST/DNA-R fusion protein was a DNA-binding molecule, a Southwestern blot was performed. The purified GST/DNA-R fusion protein and GST protein alone were electrophoresed on a polyacrylamide gel, electrophoretically transferred to nitrocellulose and then probed with biotinylated DNA. DNA binding was visualized by addition of steptavidin conjugated with horse radish peroxidase (HRP) using conventional methods. As seen in FIG. **11**, purified GST/DNA-R fusion protein (FIG. **11B**, lane 1), but not GST protein alone (FIG. **11B**, lane 2) bound biotinylated plasmid DNA. Other peptides seen to react with biotinylated DNA/streptavidin-HRP in the GST/DNA-R samples (FIG. **11B**, lanes 1) probably represent partially degraded GST/DNA-R peptides and/or traces of contaminating bacterial proteins. Lanes in Figure 11A represent no added DNA.

Third, as a final assessment of the DNA binding ability of the purified DNA receptor fragment (amino acids 1-575) the ability of the purified peptide to bind to ELISA plates coated with plasmid DNA (VARELISA dsDNA kit, Pharmacia) was determined. Binding of the DNA receptor peptide was detected using the rabbit anti-DNA-R polyclonal antisera described Example 3. As shown in FIG. **12**, purified DNA-R peptide bound to DNA coated plates when tested at both 1 μg/mL and 10 μg/mL. Negative controls not including the DNA-R fragment showed no reactivity.

These results demonstrate that the DNA receptor gene of the invention encodes a protein that specifically binds DNA, and that the DNA binding portion of the molecule resided in the protein fragment having amino acid sequence 1-575 of the native protein.

Having demonstrated that the protein encoded by the cloned cDNA of the invention bound DNA, the affinity of soluble GST-DNA-R for DNA was estimated using a nitrocellulose filter-binding assay. The assay was performed using cold DNA competition where known amounts of GST/DNA-R (2 nM) and labeled DNA (200 pM) were titrated with increasing

amounts of unlabeled calf thymus DNA. These results demonstrated that DNA binding to the DNA-R of the invention was saturable, consistent with its identification as a specific receptor. A Scatchard transformation of the data yield a $K_D \sim 4$ nM (FIG. 14).

To demonstrate that the binding of DNA by the soluble form of the DNA-R (amino acids 1-575) was not due to monospecific charge-related interactions, the role in DNA binding of the zinc finger domain at amino acids 416-435 was examined. Using site-directed mutagenesis, the codon for the conserved zinc finger cysteines at either amino acids 416 or 431 were altered to a codon for either alanine or serine. The mutagenized GST/DNA-R fusion proteins were expressed in *E. coli* and affinity purified on glutathione sepharose, then tested for their ability to bind to immobilized DNA by ELISA, all substantially as described above. Purified GST/DNA-R (1-575) fusion protein bound to ELISA plates coated with calf thymus DNA (Magiwel, United Biotech, Mountain View, CA), as shown in FIG. **13**. Mutagenesis of either cysteine 416 or 431 reduced DNA binding to approximately 50% of the level observed for wild-type GST/DNA-R fusion protein, strongly suggesting that this zinc finger domain is involved in specific DNA binding FIG. **13**.

These results demonstrated that DNA binding by the soluble DNA-R fragment is not simply a nonspecific charge related interaction, but rather is mediated by specific a DNA-binding motifs in the protein, including at least the zinc finger motif.

EXAMPLE 5

Soluble DNA-R Protein Inhibits DNA-Induced Cytokine Secretion and Blocks Binding of DNA to Cells

The presence of extracellular DNA in lung tissue of several chronic lung diseases, including cystic fibrosis, chronic bronchitis and bronchiectasis, causes or contributes to chronic inflammation of lung tissues with long-term pathological consequences. Extracellular DNA is known in the art to cause lung macrophages and other cells to release cytokines that mediate inflammation as part of the chronic symptomology of cystic fibrosis patients. As described in Example 2, the DNA-R protein of the invention is expressed in lung tissues, specifically in epithelial cells of the lung. This suggests that the DNA-R receptor protein of the invention is involved in inflammation by triggering the release of inflammation-causing cytokines. Thus, the ability of a soluble form of the DNA-R to bind DNA suggested that this protein fragment

could compete for binding extracellular DNA in cystic fibrosis patients and would be useful thereby as a therapeutic agent.

To determine if the soluble DNA-R fragment of the invention inhibits DNA-induced cytokine secretion, soluble DNA-R protein was examined for inhibition of CF-DNA-induced IL-6 release from J774 murine monocyte/macrophage cells in culture. In the absence of stimulating DNA, DNA-R did not induce IL-6 secretion (shown in Table I). DNA isolated and purified from a patient with cystic fibrosis (CF DNA) induced 611 pg/mL of IL-6 from J774 cells. When CF DNA was incubated first with DNA-R protein (10 ng/mL) and then added to J774 cells, the amount of IL-6 was reduced by 36% in the presence of the soluble DNA-R protein (10ng/mL). As a negative control, calf thymus DNA failed to induce detectable IL-6. To eliminate the possibility that cytokine release was caused by the presence of contaminating endotoxin, a Limulus amoebacyte assay was performed, and the CF DNA had < 0.25 ng/mL of contaminating endotoxin. In control experiments, this amount of LPS induced only 4 pg/mL of IL-6. In the second experiment (also shown in Table 1), contaminating endotoxin was removed from the soluble DNA-R, permitting the use of increased DNA-R concentrations. Soluble DNA-R protein (used in the range 10 ng/mL – 50 ng/mL) was incubated with J774 cells and 50 μg/mL of *E. coli* DNA. Cell-free supernatants were collected and IL-6 quantified by ELISA. In the absence of bacterial DNA soluble DNA-R did not induce IL-6 secretion. When bacterial DNA was added to the system, however, soluble DNA receptor protein inhibited IL-6 secretion in a dose-dependent manner (Table I).

TABLE I

Stimulus	Treatment	IL-6 (pg/mL)	% Inhibition
- - -	Medium	0	- - -
- - -	DNA-R (10ng/mL)	0	- - -
CF DNA (10μg/mL)	Medium	611	- - -
CF DNA (10μg/mL)	DNA-R (10ng/mL)	438	22
E. coli (10μg/mL)	Medium	1467	- - -
E. coli (10μg/mL)	DNA-R (10ng/mL)	945	36
E. coli (50μg/mL)	Medium	2390 ± 344	- - -
E. coli (50μg/mL)	DNA-R (10ng/mL)	1193 ± 128	50.1
E. coli (50μg/mL)	DNA-R (20ng/mL)	983 ± 212	58.9

Stimulus	Treatment	IL-6 (pg/mL)	% Inhibition
E. coli (50μg/mL)	DNA-R (50ng/mL)	652 ± 76	72.7
CT DNA[1]	Medium	0	- - -
LPS[2]	Medium	4	- - -

[1] CT = calf thymus DNA

[2] LPS = bacterial lipopolysaccharide (endotoxin)

To determine whether soluble DNA-R protein fragment was capable of preventing DNA binding to cells, J774 cells (5 x 10^5 cells) and YOYO labeled pGEM-DNA were incubated with either the soluble DNA-R protein fragment or control GST protein. Cells were incubated for 30 minutes at 4°C, centrifuged and washed twice with assay media, resuspended and incubated with 7-amino actinomycin D (7AAD) on ice for 20 minutes in order to assess viability. The samples were assessed for DNA binding by FACScan (Becton-Dickinson, Franklin Lanes, NJ). Results showed a dose-dependent inhibition of DNA binding to J774 cells (FIG. 15). Similar results were observed using human 293 cells. Additionally, the soluble DNA-R protein/DNA complex does not bind to the cell surface. The soluble DNA-R protein bound to DNA and is effective at preventing the association of DNA with the cell surface.

These results indicate that the soluble DNA-R fragment provided by this invention is useful for inhibiting cytokine release, and inflammation consequent thereto, by competitively binding either bacterial or mammalian extracellular DNA and reducing the amount of such DNA bound by cytokine-producing cells expressing the DNA-R of the invention.

EXAMPLE 6

DNA Binding to Cells Mediated by DNA-R

The experimental results disclosed above established that the soluble DNA-R fragment comprising amino acids 1-575 of the DNA-R of the invention was capable of binding DNA. Further experiments were performed to characterize DNA binding to the receptor, particularly whether the native receptor protein was capable of binding extracellular DNA at the cell surface, and whether binding is consistent with a receptor-mediated process.

In these experiments, A549 human lung carcinoma cells were harvested from log-phase cultures and treated with DNase and RNase to remove exogenous cell-surface bound nucleic acids. After treatment, the cells were washed with 10 mM EDTA and phosphate buffered saline

(PBS) to stop the action of DNase and RNase. The cells were then plated in V-bottom 96-well plates at 10^6 cells/well in PBS containing 1% fetal calf serum (FCS) and 1mM $Ca^{++}Mg^{++}$. YOYO-labeled pGEM4Z plasmid DNA was added at concentrations from 0 – 25 μg/mL in 0.2 mL media containing 1% FCS and 1 mM $Ca^{++}Mg^{++}$. The cells plus labeled plasmid were incubated for 30 minutes at 4°C, to minimize internalization of plasmid DNA. Upon completion of the 30 minute incubation, the cells were washed with 2X in PBS containing 1% FCS and 1mM $Ca^{++}Mg^{++}$ and resuspended in 0.3 mL of PBS. Cells were then fixed in 1% formaldehyde and cell-surface binding of plasmid DNA quantified by FACS.

The results of these experiments are shown in FIG. **16**. This representative FACS histogram demonstrates the A549 cell profiles seen when comparing cells incubated with either medium (FIG. **16**, curve on the left) or cells incubated with 5 μg/mL of YOYO/pGem4Z plasmid (FIG. **16**, curve shifted to the right). The geometric mean of the intensity is used to describe the cell populations. In this example, the geometric mean of the A549 cell population, treated with medium only, was 13 and increased to 34 when incubated with YOYO-labeled plasmid DNA.

A binding curve for A549 cells was then generated using a range of plasmid DNA from 0-25 μg/mL (FIG. **17A** and **17B**). The Y-axis of the graph in Figure **17A** represents the geometric mean of the fluorescence intensity of the cell populations in the graph. Cell surface binding of plasmid DNA to A549 cells began to show saturation at approximately 10 μg/mL of DNA. Treatment of cells with a 25-100 fold excess of unlabeled DNA significantly blocked the binding of YOYO/DNA to the cell surface (FIG. **17A**). The specific cell-surface binding to A549 cells, represented as the difference between total binding seen with excess unlabeled DNA, shows a binding curve with a characteristic saturation profile (FIG. **17B**).

Also examined were the cell-surface plasmid DNA binding profiles for a variety of tumor cell lines, including B16 murine melanoma cells, MOLT-4 human lymphoblastic leukemia cells, and the human Raji Burkitt lymphoma cells. In all cells examined, cell-surface DNA saturable binding profiles were obtained, consistent with a receptor-mediated mechanism of binding. Under optimal DNA binding conditions the percent of cells in the population capable of binding DNA above the background level as detected by FACS, ranged from greater than 70% (S49, DHL-6, MOLT-4) to less than 10% (D10.S, HUT-78, K562 and G361).

TABLE II

Cell Type	% Cells binding DNA	Lineage
S49	98	Murine T-cell lymphoma
MOLT-4	79	Human lymphoblastic leukemia
DHL-6	70	Human B-cell
A549	55	Human lung carcinoma
Dami	44	Human leukemia
B16	32	Murine leukemia
B9	21	Murine plasmacytoma
COS-7	20	African green monkey kidney cell
HBE014	20	Human bronchial epithelial cell
MO-7	16	Human leukemia
NOR-10	16	Murine muscle
J558	15	Murine plasmacytoma
RAJI	15	Human Burkitt lymphoma
HeLa	12	Human cervical cancer
SW480	12	Human colon adenocarcinoma
HUT-78	7	Human cutaneous T-cell lymphoma
K562	5	Human myelogenous leukemia
D10.S	3	Murine T cell
G361	3	Human malignant melanoma
Spleen	80	Normal mouse spleen cells

To determine if DNA binding is mediated by a cell-surface protein, the experiments were performed substantially as described after cells were treated with trypsin. Cell-surface DNA-binding of plasmid DNA on A549 cells was significantly inhibited by treatment of cells with trypsin after binding with YOYO-labeled DNA at 4°C (FIG. **18**).

Finally, the effect of divalent cations on cell surface DNA binding was examined. using B16 melanoma cells. These studies demonstrated a four-fold increase in fluorescence intensity when Ca^{++} is added to the binding media (FIG. **19**).

These results indicate that the DNA-R protein of the invention mediates cell surface binding of extracellular DNA in mammalian cells.

EXAMPLE 7

Internalization of Extracellular DNA into Cells Expressing DNA-R

The experiments described in Example 6 established that extracellular DNA was specifically bound to the DNA-R of the invention. Internalization of DNA into cells by the receptor was characterized using the following assay.

YOYO-labeled plasmid DNA was used to examine the kinetics of plasmid DNA internalization. The plasmid used in these assays was pEGFP-N1, encoding green fluorescent protein (Clontech, Palo Alto, CA). The assay required that cell surface binding of labeled DNA be distinguished from internalized plasmid DNA. This was accomplished by treatment of cells with trypsin to remove cell-surface proteins after incubation with plasmid DNA. This procedure permitted cell surface-bound plasmid DNA to be distinguished from internalized plasmid DNA, since trypsin treatment abolished cell surface bound DNA but not internalized plasmid DNA. In this assay, cells were plated in 24 well plates and incubated in culture media for 24h. Media was then removed and various concentrations (0-25 μg/mL) of YOYO-labeled pEGFP-N1 plasmid DNA were added. The cells plus plasmid DNA were incubated for various times (0.5 to 5 hours) at 37°C. Thereafter, the media was removed, cells were treated with trypsin, washed, and then fixed with 1% formaldehyde. FACS analysis was used to quantify fluorescence intensity.

B16 murine melanoma cells were examined for internalization of YOYO/DNA using the above protocol after incubation for 1, 3, and 5 hours (FIG. **20**). Internalization of pEGFP-N1 were found to be both dose- and time-dependent. An increasing amount of internalized plasmid DNA was seen with increasing dose of DNA and increasing time of incubation. Internalization of plasmid DNA by A549 cells was evaluated both with and without pre-treatment with unlabeled DNA. This assay was repeated with A549 cells, and similar results were obtained (FIG. **21**). Moreover, pre-treatment of the A549 cells with a 25-100-fold excess of unlabeled calf thymus DNA significantly inhibited subsequent internalization of plasmid DNA (FIG. **21**). Similar inhibition of internalization by pre-treatment of cells with excess unlabeled DNA was observed using a number of other cell lines (including B16, Raji, and

MOLT-4). This demonstration of saturable DNA binding and internalization indicates that the cell-surface DNA receptor of the invention mediates internalization of extracellular plasmid DNA.

Internalization of plasmid DNA was also observed to be a temperature-dependent process. Treatment of B16 cells at 4°C significantly inhibited the amount of plasmid DNA that was internalized as compared to cells maintained at 37°C (FIG. **22**).

In order to ascertain whether the amount of DNA-R expressed on the cell surface influences the extent of extracellular DNA binding or DNA internalization, binding and internalization of plasmid DNA was compared in two cell lines: human melanoma G361 and the 293 human cell line. The G361 cells bound relatively low amounts of DNA, while 293 cells bound larger amounts of plasmid DNA as assessed by the cell-surface DNA binding assay (FIG. **23**). Consistent with the binding results were the results obtained in these cells for DNA internalization, which showed that G361 cells internalized less plasmid DNA then 293 cells (FIG. **23**). These data are consistent with identification of the DNA-R of the invention as a cell surface DNA receptor protein.

EXAMPLE 8

Gene Expression of DNA Internalized by DNA-R

Conditions for transgene expression using DNA internalized by the DNA-R of the invention were developed.

The experiments described above established plasmid DNA concentrations that saturated cell-surface binding. Used the pEGFP-N1 plasmid coding for green fluorescent protein (GFP), which was used because GFP remains exclusively intracellular. FACS analysis was used to quantify GFP expression. In this assay, cells (1.25×10^5/well) were plated in 24-well plates and incubated overnight under mammalian cell culture conditions. On the next day, media was removed and the cells incubated for 3 hours at 37°C in 5% CO_2 with plasmid DNA in 0.3 mL of growth medium. DNA was then removed and fresh medium added to the cells. In some cases 0.3mL of growth medium was added to cells without removing the DNA. After 24-72 hours further incubation media was removed and cells washed once and then fixed with formaldehyde. Fluorescence intensity in the fixed cells was determined by FACS. However, no GFP expression was detected, even when using several different concentrations of pEGFP-N1 plasmid and incubation times. This result was consistently obtained, using a variety of cell

lines (A549, B16, Raji), incubation times (24-72 hours), and ranges of plasmid DNA concentrations (0.1 to 100 μg/mL). This result was obtained using cell lines that bind relatively higher levels of DNA on their cell-surface and those that bind lower levels of DNA. In positive controls, pEGFP-N1 plasmid was delivered by liposomes (Lipofectamine, Gibco-BRL, Gaithersburg, MD) and resulted in significant GFP fluorescence within 24 hours. Representative data using the B16 cell line incubated with either pEGFP-N1 alone or pEGFP-N1 delivered by liposomes shows the difference in GFP expression between these two techniques (FIG. **24**).

In view of these results, the experiments were repeated with A549 cells in the presence of nocodazol, a microtubule inhibitor. Use of this inhibitor was indicated because one possible explanation of the unsuccessful experiments is that the DNA internalized by the DNA-R of the invention had been degraded, and nocodazol treatment was expected to reduce the extent of such degradation. Treatment of A549 cells with nocodazol prior to incubation with pEGFP-N1 resulted in a significant increase in expression of GFP as compared to cells that were not treated with nocodazol and incubated with pEGFP-N1 (FIG. **25**). Cells which were not treated with nocodazol failed to demonstrate detectable expression of GFP (FIG. **25**).

These results indicated that uptake of extracellular DNA mediated by the DNA-R of the invention required additional treatment to result in expression of genes encoded therein, and the above assay provides a prototype of assays for identifying such compounds. In these assays, an amount of GFP-encoding plasmid DNA known to reliably produce detectable GFP expression is contacted with a mammalian cell expression the DNA-R of the invention at levels known to mediate efficient uptake of extracellular DNA. GFP gene expression is then assayed in the presence and absence of a test compound to detect increased gene expression in the presence of the compound.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.